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Stability and Emulsifying Capacity of Biosurfactants Obtained from Lignocellulosic Sources Using *Lactobacillus pentosus*

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Lactobacillus pentosus grown on sugars from agricultural residues produces biosurfactants with emulsifying properties that could facilitate the bioremediation of hydrocarbon contaminated sites. The biosurfactans obtained after growing *L. pentosus* cells on distilled grape marc hydrolyzates gave values of relative emulsion volume (EV) close to 50%, being stable after 72 h when gasoline or kerosene were employed. These EV values were higher than those achieved using commercial surfactin (14.1% for gasoline and 27.2% for kerosene). Moreover, assays carried out with kerosene showed that *L. pentosus* produced biosurfactants from distilled grape marc hydrolyzates with the highest stabilizing capacity value (ES) to maintain the emulsion (99%) followed by biosurfactants produced from hazelnut shell hydrolyzates (97%). These data are comparable with those obtained using sodium dodecyl sulfate, SDS (87.7%), whereas surfactin only gave an ES value of 65.4%. Consequently, this work shows that utilization of low-cost feedstock agricultural residues as substrates for producing biosurfactants/bioemulsifiers is possible thus removing obstacles for the wide-scale industrial application of biosurfactants/bioemulsifiers.

KEYWORDS: Lignocellulosic residues; *L. pentosus*; biosurfactans; bioemulsifiers; gasoline; kerosene

INTRODUCTION

The oil tanker Prestige caused a large oil spill off the Galician coast (in the Northwest of Spain) in 2002. Thousands of kilometers of coastline and more than 1000 beaches along the Spanish, Portuguese, and French coasts were polluted thus causing great damage to the local fishing industry. The accident is considered to be the greatest environmental disaster in the history of Spain. Approximately 64 000 tons of hydrocarbons were spilled in the incident. The cleanup cost of the Galician coastline is estimated to be around 2.5 billion euros, and the ecological consequences of this disaster still remain nowadays.

When oil interacts with the environment, most of the oil hydrocarbons remain on the water surface or adhered to soil particles due to their low solubility, being only low molecular weight hydrocarbons volatilized (*1*). Bognolo (*2*) pointed out that the main natural hydrocarbon removal mechanisms are photo-oxidation, evaporation, and microbial degradation, which may take years to stabilize and consequently years to remediate contaminated sites. The bioremediation of hydrocarbon contaminated soils is limited by the poor availability of these hydrophobic contaminants for microorganisms; it is therefore imperative to develop low cost processes with a high efficiency of remediation. Some authors (*1, 3*) propose the utilization of synthetic surfactants to disperse oil and accelerate its degradation. Surfactants increase the surface area of hydrophobic contaminants in soil or water, increasing their aqueous solubility and their microbial degradation. The main types of surface-active compounds are produced by microorganisms that reduce the surface tension at the air−water interface (biosurfactants) and those that reduce the interfacial tension between immiscible liquids or at the solid−liquid interface (bioemulsifiers) (*4*). Biosurfactants usually exhibit emulsifying capacity in addition to their ability to reduce the surface tension, but bioemulsifiers do not necessarily reduce surface tension (*1*). A criterion cited to identify bioemulsifiers is their ability to maintain at least 50% of the original emulsion volume 24 h after formation (*5*).

Microbial surfactans, named biosurfactants, have several advantages over chemical surfactants including lower toxicity and higher biodegradability and effectiveness at extreme temperatures or pH values (*6, 7*). Nevertheless, in spite of these advantages, biosurfactants must be cost competitive with the chemical synthesis. Das and Mukherjee (*8*) pointed out that the major obstacle on the wide-scale industrial application of biosurfactants is the high production cost coupled with a less productive rate, as compared to commercially available synthetic surfactants. If the production cost becomes competitive with the synthetic surfactants and the commercial availability of biosurfactants increases, the industrial use of biosurfactants can be expected to grow in the coming decade. Some authors proposed the utilization of genetically modified strains (*9*), although their use is restrictive. Moreover, during recent years, efforts have been directed to explore ways to reduce the biosurfactant production cost through improving the yield and

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Table 1. Composition of the Raw Materials Used in This Study[a]

residue	cellulose (%)	xylan (%)	araban (%)	acetyl group (%)	lignin (%)
distilled grape marc	10.8	7.5	2.2	1.6	50.9
hazelnut shells	23.7	19.6	0.7	4.1	40.8
walnut shells	23.0	16.0	1.2	3.8	37.4

[a] The results are expressed as percentage of the initial weight of sample on a dry basis.

the use of either cost-free or low-cost feedstock or agricultural byproducts as substrates for biosurfactants/bioemulsifiers production. Additionally, the culture medium (carbon source) and the growth conditions (pH, temperature, limiting nutrients, and trace elements) can influence the types and yields of biosurfactants (*7*). In previous works (*10*−*12*), we have reported the ability of *Lactobacillus pentosus* to produce cell-bound biosurfactants and the possibility to employ some lignocellulosic residues like vine shoot trimmings or distilled grape marc as carbon sources for this production. Nevertheless, studies are needed to prove their potential for contaminated hydrocarbon sites remediation.

The aim of this work was to evaluate the emulsifying capability of biosurfactants produced from lignocellulosic residues by *L. pentosus* to be employed for remediation of hydrocarbon contaminated sites.

MATERIALS AND METHODS

Raw Material. Samples of distilled grape marc and walnut and hazelnut shells were dried at room temperature and milled to a particle size suitable for hydrolysis treatment (ca. 1 mm). In order to carry out the chemical characterization of the raw material, aliquots from the homogenized lots were submitted to moisture determination and to quantitative hydrolysis in two-stage acid treatment (the first step with 72% H_2SO_4 (w/w) at 30 °C during 1 h and the second one after dilution to 4% H_2SO_4 (w/w) at 121 °C during 1 h) (*13*). The solid residue after hydrolysis was considered as Klason lignin. Hydrolyzates were analyzed by HPLC using an Interaction ION-300 column (mobile phase, 0.01 M H_2SO_4; flow rate, 0.4 mL/min; temperature, 50 °C; IR detection). **Table 1** shows the composition of these lignocellulosic residues.

Preparation of Hemicellulosic Hydrolyzates. Prehydrolysis (partial acid hydrolysis of the hemicellulosic fraction) of distilled grape marc was carried out in an autoclave at 130 °C with 3% H_2SO_4 acid for 30 min using a liquid/solid ratio of 8 g/g, whereas prehydrolysis of walnut and hazelnut shells were carried out at 130 °C with 3% H_2SO_4 for 15 min at the same liquid/solid ratio.

Microorganism. *Lactobacillus pentosus.* CECT-4023T (ATCC-8041) was obtained from the Spanish Collection of Type Cultures (Valencia, Spain). The strain was grown on MRS broth at 31 °C for 15 h and 150 rpm. Following this, the culture was centrifuged, and *L. pentosus* cells were resuspended in the same volume of hydrolyzate.

Lactic Acid Fermentation. Hemicellulosic hydrolyzates from agricultural residues were neutralized with powdered $CaCO_3$ to a final pH of 6.0, and the $CaSO_4$ precipitated was separated from the supernatant by filtration. The clarified liquors were supplemented with 10 g of yeast extract/L and 10 g of corn steep liquor/L (*14*), sterilized at 100 °C for 1.25 h, and used directly as fermentation media. After sterilization, 15 mL of the aqueous inoculum suspension was added to the fermentation media. Experiments were carried out at 31 °C in a 2 L Biostat B batch reactor (Braun, Melsungen, Germany) with 1.5 L of working volume at 150 rpm. During fermentation, pH was controlled at 6.5 by addition of 5 M NaOH. Samples (2 mL) were taken at given fermentation times and centrifuged at 6 000 rpm for 10 min. Supernatants were stored for sugar analysis using a high-performance liquid chromatograph (Agilent, model 1100, Palo Alto, CA) with RI detection using a Transgenomic ION-300 column (Transgenomic Inc., San José, CA) eluted with 0.02 M H_2SO_4 at a flow rate of 0.4 mL/min.

Biosurfactant Extraction and Surface Tension Determination. Because of the fact that *L. pentosus* produces biosurfactants bound to the cell wall, *L. pentosus* cells were recovered by centrifugation (4 500*g*, 30 min, 20 °C) from the fermentation media, washed twice in demineralized water, and resuspended in phosphate buffer saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.4). Following this, the PBS extracts were left at room temperature up to 2 h with gentle stirring for biosurfactant release and centrifuged to remove the bacteria. The surface-activity of biosurfactants produced by *L. pentosus* was determined by measuring the surface tension (ST) of the PBS extracts with the Ring method (*15*), using a KRUSS Tensiometer from GmbH (Hamburg, Germany) equipped with a 1.9 cm DuNoüy platinum ring at room temperature. Ring tensiometers determine the surface tension with the help of an optimally wettable ring suspended from a precision balance. In the Ring method, the liquid is raised until contact with the surface and then lowered again so that the liquid film produced beneath the ring is stretched. As the film is stretched, a maximum force is experienced, which is measured and used to calculate the surface tension. On the other hand, in order to estimate if the biosurfactans were over their critical micellar concentration (CMC), the PBS extracts containing the biosurfactants were diluted several fold until achieving the dilution ratio value (F_{CMC}) at which surface tension starts to increase. The biosurfactant concentration at this dilution ratio is known as the CMC. The ST of the nondiluted and diluted PBS extracts was also measured (at room temperature) after incubating them at different pH values (4.4, 7.4, 10.4) and temperatures (10 °C, 25 °C, 40 °C) for 24 h.

Relative Emulsion Volume and Stability of PBS Biosurfactant/Bioemulsifier Extracts from *L. pentosus* Cells. Two hydrocarbon environmental contaminants (gasoline and kerosene) were mixed in equal volume (2 mL) with PBS biosurfactant/bioemulsifier containing extracts of *L. pentosus* cells.

Once the hydrocarbons and *L. pentosus* biourfactants/bioemulsifiers were mixed and shaken vigorously for 2 s, they were left to stand for 1 h. After that time (considered the initial time: 0 h), the relative emulsion volume (EV, %) and stability (ES,%) were measured in intervals up to 72 h by using eqs 1 and 2 proposed by Das et al. (*16*):

$$\mathrm{EV}\ (\%) = \frac{\text{emulsion height (mm)} \times \text{cross section area (mm}^2\text{)}}{\text{total liquid volume (mm}^3\text{)}} \times 100 \quad (1)$$

$$\mathrm{ES}\ (\%) = \frac{\text{EV at time } t}{\text{EV at 0 h}} \times 100 \quad (2)$$

Moreover, the emulsions formed by *L. pentosus* biosurfactants/bioemulsifiers were compared with those formed by a 1% (w/v) solution of the synthetic surfactant sodium dodecyl sulfate (SDS) in deionized water, as proposed by Das et al. (*16*), and with surfactin, a commercial biosurfactant produced by *Bacillus subtilis*, at a concentration of 0.005% (w/v). These concentrations correspond approximately to 10 times the CMC for SDS (1 g/L) and surfactin (5 mg/L).

Additionally, the percentage of emulsified organic phase (EOP) was also calculated using eq 3 as follows:

$$\mathrm{EOP}\ (\%) = \frac{\text{vol TOP (mm}^3\text{)} - (\text{NEOP (mm)} \times \text{cross section area (mm}^2\text{))}}{\text{vol TOP (mm}^3\text{)}} \times 100 \quad (3)$$

where TOP is the total volume of organic phase and NEOP is the nonemulsified organic phase.

Statistical Analysis. Fermentations were made in duplicate, whereas surface tension measurements and emulsion experiments were performed in triplicate. In order to evaluate the effect of pH and temperature on the surface activity of the biosurfactant extracts, the surface tension data with replicates were fitted to a second-order polynomial with lineal interactions by mean of response surface analysis, supported by NemrodW 2000 software, and based on multiple regression and analysis of variance (ANOVA) at 5% significance level. Data of relative emulsion volume, emulsion stability, and emulsified

Table 2. Composition of Hemicellulosic Sugars in Hydrolyzates Coming from Different Agricultural Residues

residue	glucose (g/L)	xylose (g/L)	arabinose (g/L)
distilled grape marc	2.2	8.2	2.1
hazelnut shells	0.6	20.0	0.6
walnut shells	1.3	18.4	1.6

organic phase were subjected to an analysis of variance by using SPSS statistical software package, and significant treatment differences were separated by Turkey's multiple range test at $p < 0.05$.

RESULTS AND DISCUSSION

Fermentation of Hemicellulosic Hydrolyzates to Obtain Biosurfactants/Bioemulsifiers. Microbial molecules with high surface activity are classified as biosurfactants. In addition, some of these compounds can also exhibit emulsifying capacity. In this work, biosurfactants/bioemulsifiers were produced using a mixture of hemicellusosic sugars (mainly glucose, xylose, and arabinose), which were obtained after prehydrolysis of three lignocelluosic residues. **Table 2** shows the concentrations of the hemicellulosic sugars present in solutions obtained after prehydrolysis of distilled grape marc and hazelnut and walnut shells. It can be observed that walnut and hazelnut shells gave higher concentrations of sugars. However, on the basis of a previous work, although distilled grape marc rendered lower sugar concentrations, this waste represents a feedstock of sugars, which can be fermented by *L. pentosus* in order to produce biosurfactants (*11*). In the same way, biosurfactants have also been obtained after fermentation of *L. pentosus* grown on liquor-containing sugars produced by hydrolysis of vine shoot trimmings, corn cobs, barley bran, or *Eucalyptus globulus* chips (*10*).

After distillation, grape marc causes important environmental problems because usually it is discarded in the environment without additional purification treatments. This waste was chosen in the present work because of the high contaminant content and large availability in Spain, in order to know its potential as a raw material to produce biosurfactant/bioemulsifying compounds. Moreover, in order to compare and to study the influence of different substrates on biosurfactants/bioemulsifiers production, walnut and hazelnut shells were included in this work to obtain hydrolyzates susceptible to fermentation by *L. pentosus*. Walnut and hazelnut shells were chosen as carbon sources due to the fact of their higher content in fatty acids in comparison to other lignocellulosic residues, which could result interesting because in some cases biosurfactant production was related with the composition of the fermentation medium. For example, some authors (*17*) found that hydrophobic substrates like corn oil, lard, and long chain alcohols maximized biosurfactant production by *Pseudomona aeruginosa* UG2. On the other hand, Batista et al. (*4*) found that glucose was better carbon source than fructose, sucrose, or kerosene for screening surfactant and/or emulsifier-producing microorganisms, and Amezcua-Vega et al. (*18*), working with *Candida ingens*, observed that the surface tension reduction of the culture media and the total fatty acid content of the biosurfactant were modified as the media composition changed. The highest biosurfactant production obtained for these authors was reached when higher C/Fe and C/P ratios were combined. Moreover Wei and Chu (*19*) found that the addition of iron-enriched media improved the yield of surfactin by *Bacillus subtilis*. It is apparent that there is an important effect of nutritional conditions on biosurfactant production.

Figure 1 shows the kinetics of sugar consumption and biomass production during *L. pentosus* fermentation of hydrolyzates from distilled grape marc and hazelnut and walnut shells. It is known that *L. pentosus* produces cell-bound biosurfactants (*10, 11*). Consequently, during these fermentations it would be interesting to achieve high biomass concentrations. In our case, it can be observed that the maximum amount of biomass obtained in all the cases was approximately the same (**Figure 1**), even though distilled grape marc hydrolyzates contained a lower amount of glucose and xylose than hydrolyzates from walnut and hazelnut shells. Nevertheless, after 15 h of fermentation, distilled grape marc hydrolyzates produced 0.17 g of biomass per gram of sugars consumed, whereas hazelnut and walnut shells hydrolyzates had only produced 0.07 and 0.09 g of biomass per gram of sugars consumed, respectively. This fact could represent an advantage for distilled grape marc to be employed as a carbon source for biosurfactant production, compared with the other residues tested in this study.

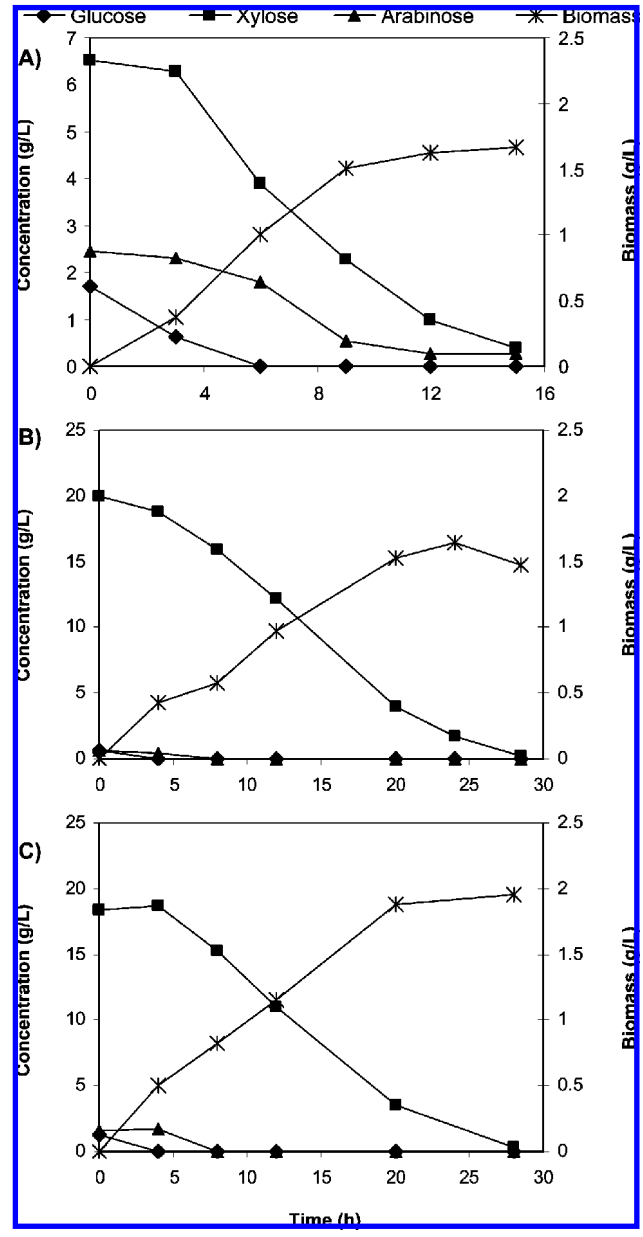

Figure 1. Kinetics of sugar consumption and biomass production during fermentation of hydrolyzates from distilled grape marc (**A**) and hazelnut (**B**) and walnut (**C**) shells.

Tensioactivity Properties of Biosurfactants/Bioemulsifiers. When a surfactant is added to air/water systems at increasing concentrations, a progressive reduction of the surface tension

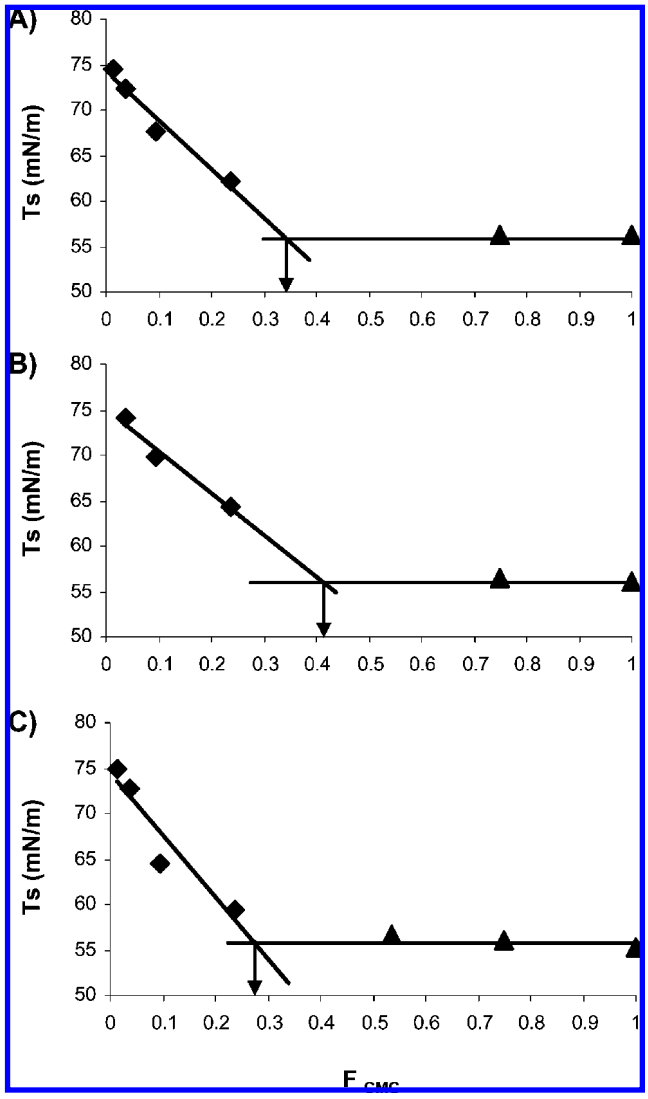

Figure 2. Dilution ratio at which the critical micellar concentrations (CMCs) were achieved for the biosurfactant/bioemulsifier PBS extracts obtained from *L. pentosus* cells grown on distilled grape marc (**A**) and hazelnut (**B**) and walnut (**C**) shell sugars.

is observed until it reaches a concentration that renders the minimum surface tension value. Above this concentration, known as the critical micellar concentration (CMC), it is not possible to continue lowering the surface tension due to the fact that surfactant molecules associate readily to form supramolecular structures such as micelles and vesicles. **Figure 2** shows the dilution ratio (F_{CMC}) at which the CMC was achieved for the biosurfactants/bioemulsifiers extracts obtained when distilled grape marc and hazelnut and walnut shell hemicellulosic were employed as sugars. It can be noted that the lowest dilution necessary for reaching the CMC corresponded to the biosurfactant extracts produced from walnut shell hydrolyzates. Therefore, it can be deduced a lower concentration of the bisurfactants/bioemulsifiers produced by *L. pentosus* growing on this raw material. Biosurfactant extracts produced when using distilled grape marc had a F_{CMC} similar to the biosurfactans extracts obtained with vine shoot trimming hydrolyzates (*10*), whereas hazelnut shells produced the biosurfactant extracts with the highest F_{CMC} (3.8).

On the other hand, in order to employ these biosurfactans for remediation of contaminated sites, it would be interesting to know the biosurfactants stability at different pH values and temperatures. **Table 3** shows the mean surface tension values of diluted above the F_{CMC} and nondiluted biosurfactant extracts measured at room temperature after incubation for 24 h at 10, 25, and 40 °C under different pH (4.4, 7.4, and 10.4). The response surface analysis of the data allowed identifying the pH as the most significant and strong assayed effect on the capacity of these extracts to reduce surface tension. The existence of quadratic positive pH terms for all the models, except that corresponding to undiluted hazelnut shell extracts, indicates the existence of a minimum, which means that lower and higher pH values will increase the surface tension, thus reflecting a loss of the extract surface activity at acidic and basic pH. The additional presence of significant negative lineal pH terms in most of the models indicates that the surface activity of these biosurfactants is negatively affected at acidic pH to a higher extent. In fact, the response surface shown in **Figure 3** for the diluted extract from hazelnut shell hydrolyzate as a representative example exhibits nearly a plateau in the interval between the neutral and basic pH assayed; this reflects that the surface activity of these biosurfactants is hardly affected by the pH in the range 7.5−10.5. The higher sensitivity of these biosurfactants to acidic pH is probably due to the presence of negative charged groups at the polar ends of the molecules (still unidentified at the moment) that are protonated under these conditions. In this sense, the characterization of the crude bound cell biosurfactant extracts produced by other Lactobacilli has revealed the presence of protein fractions (*20*) probably associated to bound phosphate (*21*), which could be also negatively affected by denaturalization at acidic pH.

In relation with temperature, the less statistical significance and the lower absolute value of the coefficients of the lineal and quadratic temperature terms with regard to the intercept, comparing to those for pH terms, indicate the low effect of these variable in the experimental domain assayed (**Figure 3**) and allow for the conclusion that these biosurfactant extracts can be considered stable in the range of temperatures between 10 and 40 °C.

Finally, the inclusion of a higher number of significant terms in all the models for the diluted extracts reflects the higher sensitivity of the system when the surfactant concentration is above the CMC.

Bioemulsifying Properties of *L. pentosus* Biosurfactans. Hydrocarbon-degrading bacteria release biosurfactants/bioemulsifiers in order to facilitate assimilation of insoluble substrates (*4, 22, 23*) Microorganisms can produce biosurfactants/bioemulsifiers in the cell-free extract or cell-bound. Biosurfactants with proven potential for remediation of contaminated sites include surfactin, produced by *Bacillus subtilis*, and the rhamnolipids from *Pseudomonas aeruginos*a (*24*−*26*).

Most of the biosurfactants produced in nature have emulsifying capacity; consequently, at this point, it would be interesting to check not only the surface activity of *L. pentosus* biourfactants but also its emulsifying capability in order to study its potential to be employed for bioremediation of hydrocarbon contaminated sites. **Figure 4A** and **B** shows the emulsion forming capacity, expressed as percentage of relative emulsion volume (EV), and stabilizing capacity, expressed as emulsion stability (ES), of biosurfactants from *L. pentosus* growing on different carbon sources. In order to compare with other surfactants, assays were also carried out with SDS and surfactin. These experiments were made using gasoline as the pollutant. After 72 h, it can be observed that, among the biosurfactants assayed, those obtained from distilled grape marc hydrolyzates produced the highest EV (42.3%), followed by the biosurfactans obtained from hazelnut (EV = 40.4%) and walnut shells (EV = 38.2%). But, the most

Table 3. Surface Tension (mN/m) Values of Diluted and Undiluted Biosurfactants from *L. pentosus*, Measured at Room Temperature after Incubation under Different pH Values and Temperatures

	pH 4.4						pH 7.4						pH 10.4					
	undiluted			diluted above CMC			undiluted			diluted above CMC			undiluted			diluted above CMC		
	25 °C	40 °C	10 °C	25 °C	40 °C	10 °C	25 °C	40 °C	10 °C	25 °C	40 °C	10 °C	25 °C	40 °C	10 °C	25 °C	40 °C	10 °C
walnut shells	59.3	60.9	61.5	75.2	73.2	75.1	53.9	57.3	55.6	64.0	62.0	65.1	56.0	56.2	56.6	68.7	61.2	61.9
hazelnut shells	54.6	60.5	59.1	72.2	68.8	72.5	55.1	57.3	54.3	60.3	61.4	61.4	54.0	55.5	55.3	61.4	58.4	59.2
distilled grape marc	56.8	60.8	58.4	71.2	74.1	73.7	55.8	57.0	57.1	63.7	67.5	67.8	56.7	58.4	59.2	62.8	61.7	63.5

interesting finding was that commercial surfactin gave the lowest EV value (only 14.1%), even being at a higher concentration than the other biosurfactants ($\sim 10 \times F_{CMC(surfactin)}$ compared to $2.4-3.8 \times F_{CMC}$ for the biosurfactants produced in this work). It means that, although surfactin is a potent biosurfactant, its emulsifying properties are not as good as those of *L. pentosus* biosurfactants produced from distilled grape marc, walnut, and hazelnut hydrolyzates. This fact could reduce the applicability of surfactin for the bioremediation of hydrocarbon contaminated sites as proposed by Mulligan et al. (*24*). Moreover, taking into account the ES values showed in **Figure 4B**, biosurfactans from agricultural residues gave similar stabilizing capacity to emulsify gasoline as SDS (around 90% of the emulsion remained stable after 72 h), whereas commercial surfactin showed poor emulsion stabilizing capacity (only 64.6 and 41% of emulsion remained after 24 and 72 h, respectively). In addition, **Figure 5A** shows the percentage of emulsified organic phase (EOP) for gasoline, where it can be observed that biosurfactants from *L. pentosus* growing on agricultural residues emulsified around 64% of gasoline in all the cases, whereas commercial surfactin only emulsified 25% of the organic phase.

In order to compare with other data found in the literature, the previous experiments were also carried out using kerosene as the organic phase. **Figure 4C** and **D** shows the relative emulsion volume (EV) and stabilizing capacity (ES) achieved using kerosene. These values were similar to those reached employing gasoline except for the biosurfactants obtained by fermentation of walnut shell hydrolyzates. In this case, the EV percentage decreased to 20.2%, which was close to that value achieved using surfactin. This different behavior among biosurfactants from *L. pentosus* could be due to the fact of differences in the composition of the biosurfactant molecules produced by this microorganism depending on the nutritional components of fermentation media, confirming the results found by other authors (*4, 18, 19*). Furthermore, in **Figure 2**, it can

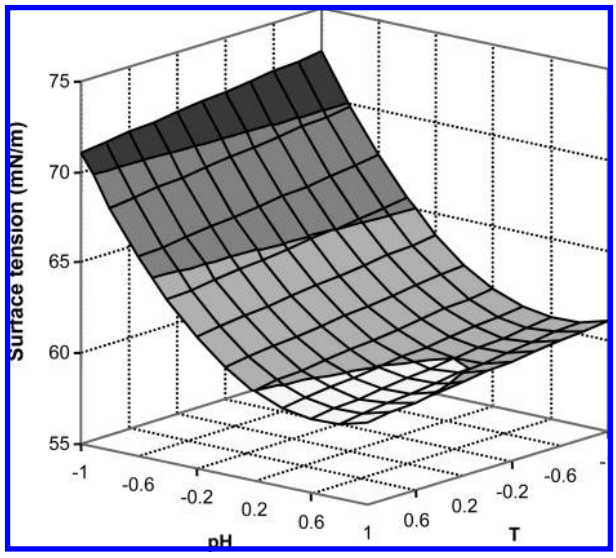

Figure 3. Response surface showing the effect of pH and temperature of extract incubation on the surface tension of the biosurfactant diluted PBS solution obtained from *L. pentosus* cells grown on hazelnut shell hydrolyzates.

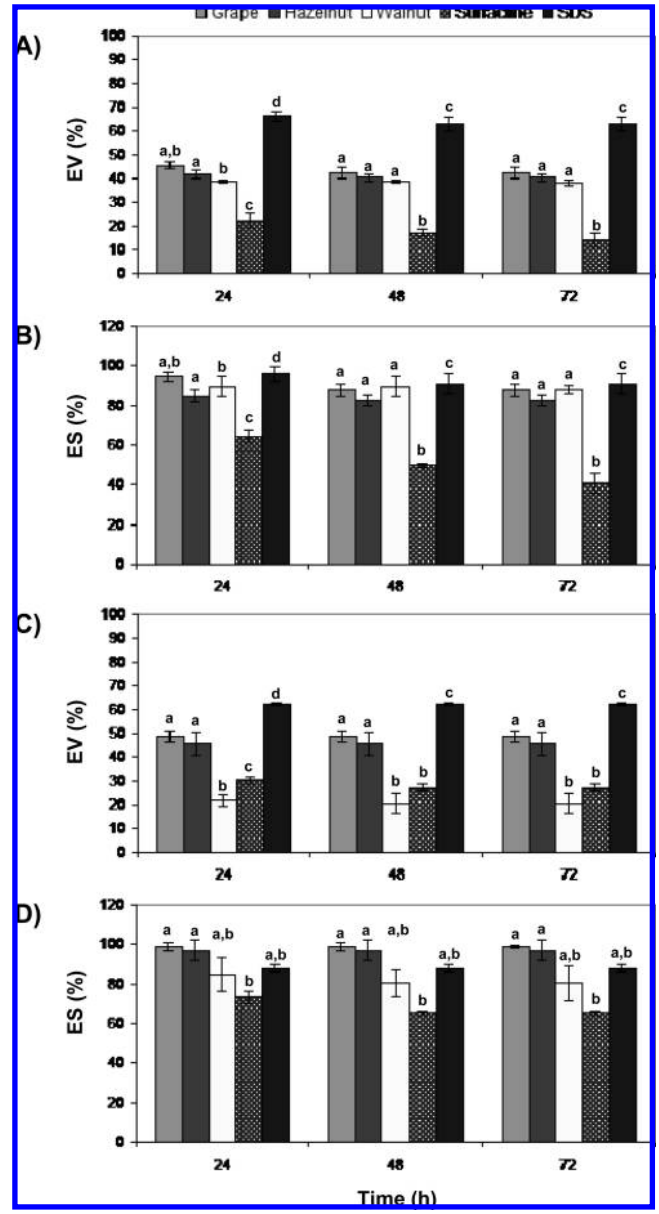

Figure 4. (**A**) Relative emulsion volume (EV) of biosurfactants from *L. pentosus* growing on different carbon sources, using gasoline. (**B**) Emulsion stability (ES) of biosurfactants from *L. pentosus* growing on different carbon sources, using gasoline. (**C**) EV of biosurfactants from *L. pentosus* growing on different carbon sources, using kerosene. (**D**) ES of biosurfactants from *L. pentosus* growing on different carbon sources, using kerosene. Different letters mean significant differences between values.

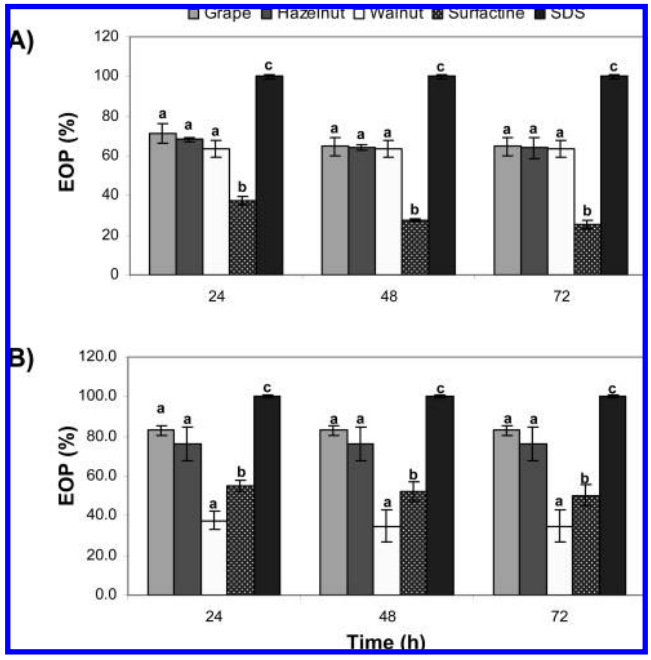

Figure 5. (**A**) Percentage of emulsified organic phase (EOP) for gasoline with different surfactants. (**B**) EOP for kerosene with different surfactants.

be noted that *L. pentosus* grown in walnut shell hydrolyzates gave a lower concentration of biosurfactans/bioemulsifiers, as deduced by its lower dilution ratio value to achieve the CMC.

Relating to the stabilizing capacity (ES) of biosurfactants from agricultural residues using kerosene, biosurfactants from distilled grape marc hydrolyzates gave the highest ES value (99%), followed by biosurfactants from hazelnut shell hydrolyzates (97%). These values are comparable with those obtained using SDS (87.7%), whereas surfactin only gave a stabilizing capability of 65.4%, which was slightly higher than the value achieved using gasoline. Batista et al. (*4*), working with bacteria isolated from petroleum contaminated sites, found that those compounds that reduced surface tension produced larger volume emulsions but with lower stability than those compounds produced by microorganisms that did not reduce surface tension. In this work, *L. pentosus* not only produces biosurfactants that reduce the surface tension but also produces large relative emulsion volumes, which are comparable with those values obtained by Batista et al. (*4*)

Figure 5B shows the percentage of emulsified organic phase (EOP) for kerosene, and it was observed that biosurfactants from distilled grape marc and hazelnut shell hydrolyzates emulsified 83.0 and 76.4% of kerosene, respectively, whereas biosurfactant from walnut shell hydrolyzates and surfactin only emulsified 34.9 and 52.1% of the organic phase, respectively. On the other hand, Menezes et al. (*23*), working with microorganisms isolated from soils contaminated with diesel oil, found that no substantial emulsification was achieved with the cell-free extracts, indicating that the emulsifying activity of those biosurfactans was not extracellular.

In conclusion, biosurfactants produced by *L. pentosus* from distilled grape marc and hazelnut shell hydrolyzates exhibited a higher emulsion-stabilizing capacity than surfactin, maintaining close to 50% of the original emulsion volume for 72 h. Additional studies would be needed for the direct application of these biosurfactants to contaminated hydrocarbon sites.

LITERATURE CITED

(1) Karanth, N. G. K.; Deo, P. G.; Veenanadig, N. K. Microbial production of biosurfactant and their importance. *Ferment. Sci. Technol.* **1999**, *77*, 116–126.
(2) Bognolo, G. Biosurfactants as emulsifying agents for hydrocarbons. *Colloids Surf., A* **1998**, *152*, 41–52.
(3) Iqbal, S.; Khalid, Z. M.; Malik, K. A. Enhanced biodegradation and emulsification of crude oil and hyperproduction of biosurfactans by gamma ray induced mutant of *Pseudomonas aeruginosa*. *Lett. Appl. Microbiol.* **1995**, *21*, 176–179.
(4) Batista, S. B.; Mounteer, A. H.; Amorim, F. R.; Totola, M. R. Isolation and characterization of biosurfactant/bioemulsifier-production bacteria from petroleum contaminated sites. *Bioresour. Technol.* **2006**, *97*, 868–875.
(5) Willumsen, P. A. E.; Karlson, U. Screening of bacteria, isolated from PAD-contaminated soils, for production of biosurfactant and bioemulsifiers. *Biodegradation* **1997**, *7*, 415–423.
(6) Kosaric, N. Biosurfactants in industry. *J. Am. Oil Chem. Soc.* **1998**, *64*, 1731–1737.
(7) Cameotra, R.; Makkar, R. Synthesis of biosurfactants in extreme conditions. *Appl. Microbiol. Biotechnol.* **1998**, *50*, 520–529.
(8) Das, K.; Mukherjee, R. K. Comparison of lipopeptide biosurfactants production by *Bacillus subtilis* strains in submerged and solid state fermentation systems using a cheap carbon source: some industrial applications of biosurfactans. *Process Biochem.* **2007**, *42*, 1191–1199.
(9) Martins Das Neves, L. C.; Miazaki Ohara Miyamura, T. T.; Junji Kobayashi, M.; Vessoni Penna, T. C.; Converti, A. Production of biosurfactant by a genetically-modified strain of *Bacillus subtilis* expressing green fluorescent protein. *Ann. Microbiol.* **2007**, *57*, 377–381.
(10) Moldes, A. B.; Torrado, A. M.; Barral, M. T.; Domínguez, J. M. Evaluation of biosurfactant production from various agricultural residuees by *Lactobacillus pentosus*. *J. Agric. Food Chem.* **2007**, *55*, 4481–4486.
(11) Portilla Rivera, O. M.; Moldes, A. B.; Torrado, A. M.; Domínguez, J. M. Lactic acid and biosurfactants production from hydrolyzed distilled grape marc. *Process Biochem.* **2007**, *42*, 1010–1020.
(12) Rodrigues, L.; Moldes, A.; Teixeira, J.; Oliveira, R. Kinetic study of fermentative biosurfactant production by *Lactobacillus* strains. *Biochem. Eng. J.* **2006**, *28*, 109–116.
(13) Vázquez, D.; Lage, M. A.; Parajó, J. C.; Vázquez, G. Transformación de materiales lignocelulósicos: Composición, fraccionamiento y aprovechamiento. *Rev. Agroquim. Tecnol. Aliment.* **1991**, *31*, 143–164.
(14) Téllez-Luís, S.; Moldes, A. B.; Alonso, J. L.; Vázquez, M. Optimization of lactic acid production by *Lactobacillus delbrueckii* through response surface methodology. *J. Food Sci.* **2003**, *68*, 1454–1458.
(15) Kim, S.; Lim, E.; Lee, S.; Lee, J.; Lee, T. Purification and characterization of biosurfactants from Nocardia sp. L-417. *Biotechnol. Appl. Biochem.* **2000**, *31*, 249–253.
(16) Das, M.; Das, S. K.; Mukherjee, R. K. Surface active properties of the culture filtrates of a *Micrococcus* species grown on *n*-alkanes and sugars. *Bioresour. Technol.* **1998**, *63*, 231–235.
(17) Mata-Sandoval, J. C.; Karns, J.; Torrents, A. Effect of nutritional and environmental conditions on the production and composition of rhamnolipids by *P. aeruginosa* UG2. *Microbiol. Res.* **2001**, *155*, 249–256.
(18) Amezcua-Vega, C.; Poggi-Varaldo, H. M.; Esparza-García, F.; Ríos-Leal, E.; Rodríguez-Vázquez, F. Effect of culture conditions on fatty acids composition of a biosurfactan produced by *Candida ingens* and changes of surface tension of culture media. *Bioresour. Technol.* **2007**, *98*, 237–240.
(19) Wei, Y. H.; Chu, I. M. Enhancement of surfactin production in iron enriched media by *Bacillus subtilis* ATCC 21332. *Enzyme Microb. Technol.* **1998**, *22*, 724–728.
(20) Howard, J. C.; Heinemann, C.; Thatcher, B. J.; Martin, B.; Gan, B. S.; Reid, G. Identification of collagen-binding proteins in *Lactobacillus* spp. with surface-enhanced laser desorption/ioniza-

tion-time of flight proteinchip technology. *Appl. Environ. Microbiol.* **2000**, *66*, 4396–4400.
(21) Rodrigues, L. R.; Teixeira, J. A.; van der Mei, H. C.; Oliveira, R. Physicochemical and functional characterization of a biosurfactant produced by *Lactococcus lactis* 53. *Colloids Surf., B* **2006**, *49*, 79–86.
(22) Deziel, E.; Paquette, G.; Villemur, R.; Lepine, F.; Bisaillon, J. G. Biosurfactant production by a soil Pseudomonas strain growing on polycyclic aromatic hydrocarbons. *Appl. Environ. Biotechnol.* **1996**, *62*, 1908–1912.
(23) Menezes, F.; Oliveira, F. A.; Okeke, B. C.; Frankenberger, W. T. Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. *Microbiol. Res.* **2005**, *160*, 249–255.
(24) Mulligan, C. N.; Yong, R. N.; Gibas, B. F. Surfactant enhanced remediation of contaminated soil: a review. *Eng. Geol.* **2001**, *60*, 371–380.
(25) Wang, Q.; Fang, X.; Bai, B.; Liang, X.; Shuler, P. J.; Goddard, W. A., III; Tang, Y. Engineering bacteria for production of rhamnolipid as an agent for enhanced oil recovery. *Biotechnol. Bioeng.* **2007**, *98*, 842–853.
(26) Das, K.; Mukherjee, A. K. Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. *Bioresour. Technol.* **2007**, *98*, 1339–1345.

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